# INFLUENZA STUDIES

## II. A SEARCH FOR OBLIGATE ANAEROBES IN RESPIRATORY INFECTIONS. AN ANAEROBIC MICROCOCCUS *

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This is a report of an investigation carried out in connection with the work on respiratory diseases at the University of Chicago during the late winter and spring of 1920.

Cultures were made of 33 specimens taken from 25 persons suffering from inflammatory conditions of the upper respiratory tract, chiefly rhinitis, simple or complicated with pharyngitis, tonsillitis, or bronchitis. Six of the specimens came from patients supposed to have become ill with influenza from 4 to 9 days previosuly, but there is unsatisfactory clinical proof of influenza in all these cases. Rhinitis cases were selected especially for this study in order, if possible, to confirm the interesting observations of Tunnicliff on an anaerobic spirochete [1] cultivable from infections of the accessory sinuses, and on B. rhinitis.[2] The fixation experiments of Howell [3] seemed to support the idea of etiologic relationship in those cases in which the latter organism was found. Tunnicliff also found an obligate anaerobe, nonpathogenic for guinea-pigs and of doubtful identity, in a case of chronic bronchitis.[4]

This paper has nothing to do with a separate study of the possibility of cultivating a filtrable virus by anaerobic means from the same materials, which is considered in another article.

The material used for cultures consisted generally of nasal washings in 40 c c of Ringer's solution, but in a few cases sputum and swabs were utilized. An effort was made to secure the specimens as soon as possible after the onset of the "cold," but it was often several days

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[1] J. Infect. Dis., 1913, 13, p. 280.

[2] Ibid., p. 283; 1915, 16, p. 493.

[3] Ibid., p. 456.

[4] Ibid., 1913, 13, p. 289.

before the patients, mostly students, presented themselves for examination. Cultures were made at once after the specimen was secured.

No attempt was made in the beginning to duplicate exactly the technic of Tunnicliff in view of certain fallacies that are believed to attend all surface methods of isolation when applied to obligate anaerobes. The argument concerning this matter has been presented in another place.* Suffice it to say, that my preference, after extensive experience in the use of a modified Wright's method of surface isolation of obligate anaerobes, is for deep glucose agar. Dilutions of the original material without enrichment were made so as to provide well separated colonies in the depths. The tubes were broken open usually after from 2 to 3 days' incubation at 37 C., the agar pushed out into a sterile Petri dish, sliced with a boiled knife, seared with a hot spatula, and the underlying colony removed entire to freshly boiled and cooled deep brain medium. No colonies were picked from within 2 cm. of the upper surface of the agar. As a rule, three colonies were transferred, except when a variety of form indicated a larger number of bacterial types. Incubation of such transferred colonies usually resulted in vigorous growth within 24 hours, but in a few cases no growth could be secured. These may have been organisms for which the cultural conditions were unsuitable, yet the most fastidious of the sporulating anaerobes develop well under such circumstances. When growth became apparent, either by turbidity or gas production or both, a transfer on the surface of an agar or blood-agar slant for incubation under aerobic conditions was made. By this means all the cultures isolated during the early part of the investigation were shown to be facultative aerobes. Their further identification was then effected in the usual way.

When it became apparent that no obligate anaerobes could be recovered by the method of deep colony isolation steps were taken to duplicate the technic of Tunnicliff in all its details. We were unable to secure goat blood, however, and had to substitute in various experiments that of horse, sheep or rabbit. The agar used was slightly alkaline to phenolphthalein as in her procedure, although as a rule, agar to which blood is added should be adjusted more nearly to the neutral point, i. e., $P_H$ 7, in order to avoid spontaneous hemolysis. Streaked in the usual way for isolation the blood agar slants were rendered anaerobic (degree of anaerobiosis uncertain and probably variable) by pushing in the absorbent cotton stoppers, loading with pyrogallic acid, adding 1-2 cc of 10% lye and stoppering with a

* J. Infect. Dis., 1920, 27, p. 577.

rubber stopper. The cultures so prepared were incubated at 37 C. in an inverted position to avoid soiling the slant with lye. Open as this procedure is to objections, it provides one of the best of the surface culture methods for anaerobes; this method happened to be one with which I was thoroughly familiar. The latter part of the investigation thus involved both deep and surface methods of isolation.

In addition to the difference in cultural methods used at first, it had been my practice to pick the colonies earlier than was Tunnicliff's custom. A number of the cultures, both in deep glucose agar and on blood agar, were allowed to incubate for periods varying from 5 days to a month. In only 3 cases with deep glucose agar did new colonies appear after 72 hours, and these all turned out to be hay bacilli; with blood-agar surface cultures a tardily growing obligate anaerobe was isolated, but it proved to be a coccus. With both deep glucose agar and blood agar incubated so long, a large proportion of the picked colonies refused to grow in subcultures.

Not only were the subcultures studied carefully; in every case in which the surface culture method of isolation was used, a microscopic examination of a slide from the mixed portion of growth stained by dilute carbol fuchsin for organisms similar in morphology to those described by Tunnicliff, was made. None was found.

Even with the above close approximation to the technic of Tunnicliff, none of the cultivated forms resembled those described by her; all save one of the isolated forms were facultative aerobes. The summarized data showing the incidence of the facultative aerobes isolated from anaerobic cultures are displayed in table 1.

The rather prominent incidence of various gram-negative nonsporulating rods is emphasized. The possibility of relating some of these to the organisms, found in empyema by Gordon,[5] was considered by him in his work in this laboratary, but with negative results except in a single instance. The frequency of B. coli is partly attributable to a single case (C 627) examined on four different occasions by the deep culture method, which yielded this organism each time. This was a supposed case of influenza complicated by rhinitis although there was no leukopenia; the result of the four examinations is set forth in detail:

4th day of disease.....B. coli, streptococcus.
5th day.....B. coli.
11th day.....B. coli, Staphylococcus albus.
12th day.....B. coli, Streptococcus, Staphylococcus albus, hay bacillus, unidentified coliform rod.

[5] J. Infect. Dis., 1920, 26, p. 29.

TABLE 1

SPECIMENS FROM CASES OF RESPIRATORY DISEASE YIELDING FACULTATIVE AEROBES FROM ANAEROBIC CULTURES

| | Deep Culture Isolation Only | Surface Culture Isolation Only | Both Deep and Surface Isolation | | | Total |
|---|---|---|---|---|---|---|
| Specimens | 20 | 7 | 6 | | | 33 |
| | | | Deep Only | Surface Only | Both | |
| Initial failure | 1 | 2 | — | — | — | 3 |
| B. proteus | 2 | — | — | — | — | 2 |
| B. coli | 6 | — | — | — | — | 6 |
| Intermediate* | 3 | 1 | — | — | — | 4 |
| Unidentified coliform | 1 | — | — | — | — | 1 |
| Hay bacillus | 3 | — | — | — | — | 3 |
| Diphtheroid | 3 | 1 | — | — | — | 4 |
| Gram negative diplococci | — | 1 | 1 | — | — | |
| M. tetragena | 1 | — | — | — | — | 1 |
| Streptococcus | 5 | 3 | 1 | 2 | 1 | 12 |
| Staphylococcus albus | 4 | 1 | 3 | 1 | 3 | 12 |
| Staphylococcus aureus | 1 | — | — | 1 | — | 2 |

* Gram negative rods, nonsporulating, fermenting glucose, but having no digestive action on lactose and gelatin.

*An Anaerobic Micrococcus.*—In one instance, a 48-hour rhinitis (C 381), an obligate anaerobic coccus, was recovered and isolated. It was associated with an unidentified coliform rod and with Staphylococcus albus. It was isolated from the surface of a blood-agar slant cultivated at 37 C., in which anaerobiosis was secured by the modified Wright method described. After four days' incubation, only one large white colony was present on the surface of the slant; it proved later to be Staphylococcus albus. On the sixth day, several tiny pin-point colonies in addition were present; they were picked to deep brain medium. The deep glucose agar in this case was markedly split by gas on the second day of incubation, and on the fourth the tube was broken open for the isolation of the well separated colonies in the agar; they were all found to be the coliform rod referred to. The slow growing nature of the coccus, since proved to develop well in deep glucose agar, precluded its isolation from the deep agar, under the circumstances.

The deep brain cultures of the picked colonies were incubated at 37 C. for several days. Growth was doubtful at first, but after a time became evident by the turbidity of the supernatant liquid. Staining was unsatisfactory from brain medium because of the natural detritus present and the small size of the organisms. Subcultures to plain agar and blood agar incubated aerobically have failed consistently to show any growth. It may be remarked that in this investigation and elsewhere I have frequently recovered anaerobically streptococci that failed to develop aerobically in the primary culture but did so in subsequent cultures. Numerous subcultures of the present organism have

been secured in brain medium, in the constricted tube with marble seal using broth with and without glucose, on blood agar under pyrogallic acid and alkali, and in the depths of glucose agar, but never on the same medium under aerobic cultivation.

The morphology is best seen in broth cultures. The organism is a minute, nonmotile coccus, occurring singly, in pairs, and in small clumps. Chains were not observed. It is gram-positive and stains readily with dilute carbol fuchsin but not so well with Loeffler's alkaline methylene blue. It is not acid fast.

Growth in all mediums is somewhat slow and distinctly better at 37 C. than at room temperature. In peptone meat infusion broth in the constricted tube with marble seal[6] a slight homogeneous turbidity appears below the marble in 24 hours at 37 C.; in 48 hours the broth is likely to be distinctly turbid above and below the seal. In such a medium, with Armour's peptone, which I have used simultaneously for demonstrating indol production by B. tetani and certain other obligate anaerobes, no indol is produced by this organism in four days' incubation. The presence of glucose in broth induces a somewhat more rapid growth. No gas is formed, but glucose is fermented with the liberation of acid. The fermentation of other carbohydrates has not been tested.

Growth in deep brain medium yields neither gas nor discoloration of the tissue.

No growth could be secured in a few trials on plain and glucose agar slants under alkaline-pyrogallol. Blood-agar slants by this method yield a delicate spreading film almost invisible, or minute bead-like colonies. Such growth suspends readily in salt solution.

Uniform heavy seeding of melted deep glucose 1 per cent. agar gives in 48 to 96 hours a band of fine colonies about 1 cm. from the surface. Longer incubation usually thickens this band from about 1 mm. to 3 or 4 mm. and often duplicates it with a thinner one a little deeper in the medium, suggesting Liesegang's rings. Fairly well separated colonies have been observed to develop in the depths of such heavily seeded cultures after a week or ten days. While these observations are by no means unique in the author's experience, such an occurrence is not common. There is no evidence in this instance that it is in any way dependent on impurity of the culture, which might be a reasonable speculation in the case of the less rare sporulating anaerobic rods. No gas is formed in glucose agar.

Light seeding of deep glucose agar seems not to predispose to ring formation to the same degree. Well separated colonies appear in the depths in 2 or 3 days, at first minute, but gradually increasing in size up to a millimeter at the end of a week. They are all dense, compact and opaque; some are flat with a leaf-like projection from the middle of one side; some are like 3 blades joined at equal angles of 120 degrees, and some are formed of 4 blades, giving the appearance of a cross when looked at from the proper direction. These variations appear not to have any special significance. Subcultures from isolated colonies show the same variations in colony form. This anaerobe appears not to have any action on milk. No growth could be secured in gelatin.

There is no evidence of pathogenicity for guinea-pigs or rabbits.

A guinea-pig, weighing 240 gm., was injected with 1 c c of a 4-day glucose-broth culture prepared in the constricted tube with marble seal. There was no result during a week's observation, at the end of which time the animal had gained 26 gm. in weight.

A rabbit, weighing 2500 gm., was injected intravenously with 1 c c of a 5-day plain broth culture from a constricted tube. There was no harmful effect on this animal.

[6] Hall: Univ. Calif. Public. in Path., 1915, 2, p. 147.

A normal rabbit, weighing 1850 gm., and a guinea-pig, weighing 290 gm., were each perfused with 1 c c of a 3-day glucose broth culture in the right nasal passage. No symptoms of discomfort or respiratory disease appeared during the week immediately following.

The cultures used were in every case active vigorous suspensions of moderate turbidity.

Obligately anaerobic cocci have not been described often. Jungano and Distaso [7] quote some references, but the writer has no access to the work of these authors at present. Wehrsing and Marwedel [8] found an obligate anaerobic streptococcus forming gas (!!) in a war wound. It was described as blackening blood broth and presenting a putrid odor, and was thought to be identical with Streptococcus putridus of Schottmüller. Weinberg and Seguín [9] consider that Wehrsing and Marwedel probably had to do with a mixed culture containing some gas forming anaerobe difficult of isolation. This supposition is supported by the fact of gas production which would be unusual for a coccus.

It is impossible to assign our organism definitely at this time. Morphologically, it resembles the staphylococci; culturally, it resembles the streptococci.

## SUMMARY

No organisms resembling those described by Tunnicliff were seen in the cultures examined in this series. The results seem more comparable to those recorded by Norton,[10] who failed to find any obligate anaerobes in the cultivation by anaerobic methods of sputum, nasopharyngeal swabs, and blood from influenza patients.

The probability of obligate anaerobes developing in the respiratory tract seems slight in view of the excessive air supply, and the above findings indicate that such infections occurred, if at all, only infrequently in the cases of respiratory disease presented for examination during the winter and spring season of 1920.

There is nothing to show that the obligate anaerobic coccus found in one instance was pathogenic.

[7] Les Anaerobies, 1910.
[8] München. med. Wchnschr., 1915, 62, p. 1023.
[9] La gangrène gazeuse, 1918.
[10] Am. Jour. Public Health, 1919, 9, p. 593.